Current Status of Bacterial Endotoxins*

Despite progress, there are gaps in understanding sepsis and developing specific therapies to combat it

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One century ago, Pfeiffer coined the term "endotoxin" to describe a biologically active heat-stable material in culture filtrates of gram-negative bacteria. According to chemical studies during the past several decades, endotoxin or lipopolysaccharide (LPS) is a complex macromolecule that contains lipid covalently linked to polysaccharide. Although the lipid component of LPS, lipid A, is critical for most biological activities of endotoxin, the polysaccharide also contributes.

Host responses to endotoxin were once viewed as an open-ended array of cellular, pathologic, physiologic, and pharmacologic activities. We now realize that this bacterial product induces the production and release of immunologically active cytokines and other mediators of the host inflammatory response. The mononuclear phagocyte, endothelial cell, and polymorphonuclear leukocyte are primary targets, although other cells (such as epithelial cells and platelets) may also participate. Specific receptor molecules and pathways of signal transduction in the host are being characterized.

Before the advent of hospital critical care units, 90% of patients with gram-negative bacteremia and septic shock died. Aggressive antimicrobial therapy, intensive cardiopulmonary support, and direct monitoring of vital signs have significantly reduced mortality. Still, about 300,000 cases of sepsis occur annually in the United States, with approximately 40% of septic patients subsequently developing shock. Past gains in reducing morbidity and mortality from sepsis and shock may now be eroding because of medical practices that rely on broad-spectrum antibiotics, immunosuppressive therapies, and invasive devices. An aging population, often with underlying chronic diseases, also adds to the difficulty of reducing the incidence of sepsis and shock. Future reductions will likely have to come from specific interventions at the molecular and genetic levels.

Despite improvements in antibiotic therapy, sepsis and septic shock have increased in frequency during the past 50 years. Although efforts to intervene therapeutically to reduce deaths from sepsis have led to substantial successes in experimental models of gram-negative bacteremia and endotoxemia, recent clinical trials have not fulfilled the early promise suggested by these animal models. Participants at a workshop, convened by the National Institute of Allergy and Infectious Diseases in July 1993, outlined current research efforts to better understand and improve these disappointing clinical results.

No magic bullet is likely to emerge from current research efforts. The dynamic interplay among LPS, cytokines, receptors, and other plasma components

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suggests that multiple agents will be required to simultaneously block different sites in the cytokine-LPS cascade. The peak-and-valley profiles of many cytokines and the rapid clearing of bloodstream LPS also demonstrate the need for rapid diagnostics to identify appropriate therapies. Without improvements in diagnosis, future clinical trials with multiple agents will be difficult to assess and may hasten the abandonment of potentially useful therapies.

Endotoxin Chemistry

Studies with synthetic lipid A reproduce precisely the in vivo and in vitro effects of purified natural lipid A, removing any lingering belief that biological activity results from some non-LPS contaminant. Studies with synthetic lipid A variants are leading to a more precise understanding of structure-function relationships.

Such studies indicate that the biological activities of LPS sometimes differ from those of the purified lipid A—perhaps because of differences in solubility of LPS and lipid A, the presence of non-LPS microbial constituents, or the polysaccharide constituents. Besides differences in the physicochemical properties of LPS and lipid A, non-LPS constituents in LPS preparations (sometimes at very low concentrations, e.g., 0.1%) also have distinct biological activities and can complicate interpretation of experiments. For example, endotoxin derived in a clinical setting likely contains such contaminants, and they may play a role in models of the pathogenesis of gram-negative sepsis.

Nearly 300,000 cases of sepsis occur annually in the United States, with approximately 40% of patients developing shock.

The contribution of inner core polysaccharide components of LPS to biological activity has long been debated. Even though lipid A may be the biologically active "endotoxic center" of LPS, components of the LPS may play an independent or costimulatory role. For example, the sugar derivative KDO can stimulate interleukin-1 (IL-1) production in human and mouse monocytes and affects the production of arachidonate metabolites in mouse macrophages.

When LPS is released from the surface of gram-negative cells, it interacts with one or more serum or plasma proteins and then binds to specific LPS receptors on host cells, triggering production and release of proinflammatory mediators. Whether the released form of LPS is a more potent stimulus for mononuclear phagocytes and other key target cells than is the bacterially bound LPS is not known. Some researchers continue to question whether antibiotic-mediated release of endotoxin might serve to exacerbate the sepsis syndrome. For example, different antibiotics vary significantly in their capacity to mediate the release of LPS from gram-negative organisms growing in vitro. Moreover, differences in survival of mice with bacteremia correlate with endotoxin-releasing potential of two cell wall-active antibiotics. Whether such differences will prove clinically relevant is not known.

Mechanisms of Cellular Recognition of Endotoxin

When LPS complexes to LPS-binding protein (LBP), they bind efficiently to either soluble CD14 (sCD14) or membrane-bound CD14 (mCD14) receptor and activate cell responses. This recognition of LPS at the plasma membrane is required for LPS uptake and for induction of a transmembrane signal leading to cell activation. Although several proteins may be involved, only one of them, CD14, apparently presents LPS to additional membrane proteins that comprise a functional LPS receptor. The sCD14 pathway controls responses of endothelial and epithelial cells (EC) to LPS. The interaction of LPS with mCD14 at the picomolar concentrations likely to be encountered in sepsis is crucial for responses of cells of monocytic origin or neutrophils (MO) to LPS.

Whether additional membrane proteins serve as transducing receptors for lipid A or form part of an LPS receptor molecular complex on MO or EC is not known. Recent studies support a potentially important role for protein tyrosine phosphorylation in LPS-induced cell activation via the LBP/CD14-dependent pathway. However, the protein tyrosine kinases/phosphotyrosine phosphatases involved in protein phosphorylation and dephosphorylation, the phosphorylated substrates, and the signalling that leads to this modulation have not been identified.

Cytokines in Septic Shock

Because cytokines play a dominant role in mediating the sepsis syndrome, they provide opportunities for therapeutic intervention. IL-1 and tumor necrosis factor (TNF) are two of the key cytokines generated by endotoxin-stimulated monocytes and macrophages. These two cytokines affect nearly every cell type by increasing the expression of genes associated with the promotion of local and systemic inflammatory processes, including genes for cyclooxygenase and inducible nitric oxide synthase, and increasing expression of endothelial adhesion molecules. IL-1 and TNF synergistically induce vasodilation and leukocyte-mediated tissue necrosis, contributing to organ failure and death. In humans, because intravenous injection of either IL-1 or TNF mimics the septic shock syndrome, several approaches to modulating these cytokines are now being tested for the ability to alleviate septic shock. These include reduced synthesis of IL-1, inhibition of the IL-1 converting enzyme that cleaves pro-IL-1β, blocking antibodies to the IL-1 receptor type I, the IL-1 receptor antagonist (IL-1Ra), and soluble (extracellular) type I and type II IL-1 receptors. Therapeutic strategies for blocking TNF include reduction in synthesis, adding neutralizing antibodies to TNF, and flooding the system with soluble receptors for TNF.

Although one of the most potent agents defined to date for inducing IL-1 and TNF is endotoxin, exotoxins from gram-positive bacteria and some fungal products can also stimulate synthesis of these cytokines. Nevertheless, 25 to 50 pg of LPS per ml produces IL-1 and TNF in freshly obtained human blood monocytes. Comparable levels of endotoxin are measured in patients with septic shock. Moreover, in human volunteers, injections of 3 ng of *E. coli*-derived LPS per kg dramatically increase circulating TNF from less than 5 pg/ml to 750 pg/ml. Activated complement enhances the ability of LPS to stimulate IL-1 and TNF.

Increased levels of IL-1β, TNF, and IL-6 correlate with mortality in patients with septic shock. In these patients, IL-6 is the most consistently elevated cytokine and, of the three cytokines, the one most closely associated with mortality. However, because IL-6 by itself does not induce the septic shock syndrome in humans, this cytokine is considered a marker of the inflammatory state, rather than a cause of the syndrome.

Technical difficulties likely account, in part, for the variable results of circulating cytokine measurements in some reports, particularly in measuring IL-1β. However, even when improved methods are employed, severely ill patients with septic shock but relatively low levels of circulating IL-1β or TNF are encountered. Although the investigator may obtain the blood sample at a seemingly appropriate time, it may not correspond to the time of maximal circulating IL-1β or TNF. IL-6 production, on the other hand, is more sustained and continues to rise with severity of disease, whereas IL-1β and TNF have a "peak and valley" pattern. Nutritional deficiencies and cytokine-binding proteins are additional factors that affect the discordance between cytokine levels and the clinical state of some patients.

The strongest evidence for IL-1 or TNF contributing to the death of patients with septic shock is their improved survival when receptors for either or both of the cytokines are blocked. Nevertheless, measuring cytokine levels rapidly during the course of septic shock is valuable. For example, in a recent trial using anti-TNF monoclonal antibodies, increased survival was reported only in patients with the highest levels of circulating TNF. In another trial using IL-1Ra, a statistically significant improvement in survival was observed in a subgroup of patients with the highest risk of death.

Identification of patients with the highest levels of either TNF or IL-1β might therefore predict which patients would most benefit from anti-TNF or IL-1 receptor blockade therapy. Such information in turn might improve study design as well as reduce the possibility that a potentially useful anti-septic shock therapy might be abandoned. However, clinical septic shock does not provide the clinician with the luxury of waiting 24 h for the results of cytokine level determinations.

Alternatively, investigation may focus on IL-1β and TNF gene expression, which can be analyzed in less than 4 h by PCR using small amounts of whole blood. If this analysis could be coupled to a clinical evaluation score, the design of anti-cytokine therapy trials could be improved considerably. Animal studies document the disadvantage of late treatment with anti-cytokine-based therapies, and humans being entered late into an anti-cytokine intervention trial are likely to be at a similar disadvantage. The challenge for the next round of clinical trials is the rapid identification of those patients who will likely benefit from TNF or IL-1 blockade treatment.

Pathophysiologic Effects of LPS: Influence of Effector Mechanisms

LPS causes permeability and other changes in the cardiovascular system, including the heart, lungs, and blood vessels. Relatively little is known about the effects of minute, nonlethal doses of LPS on these tissues. LPS apparently impairs the physiologic reserves of the heart without causing discernible dysfunction. Whether LPS acts directly on the cardiovascular system, or secondarily through the release of cytokines such as TNF is not known.

LPS may have well-defined local effects on other tissues as well. For example, its effects on the liver are extensively studied. The liver is a heterogenous organ, consisting of parenchymal and nonparenchymal cells. LPS perturbs Kupffer cells, liver endothelial cells, and hepatocytes. With regard to glucose metabolism, LPS affects translocation, glucose transport, hexose monophosphate shunt activity, NADPH production, and free radical generation.

LPS has major effects on macrophages, even in relatively small concentrations. The interactions among various mediators (cytokines, eicosanoids, platelet-activating factor) are still to be elucidated. Moreover, the metabolic behavior of macrophages differs significantly depending on the anatomical location from which these cells are isolated. Whether there are similar functional differences with respect to LPS activation has not been established.

Although high levels of LPS affect lymphocyte and endothelial cell physiology, whether these influences occur at physiological concentrations of LPS is not known. Factors such as LBP or soluble CD14 may well be critical for effective interactions. For instance, endothelial cells in small blood vessels function differently from those found in large vessels, and the relative effect of these differences on LPS functions requires further investigation.

Our understanding of local effects of LPS in other tissues is even more incomplete. For example, the effect of LPS in the lung is compartmentalized; thus, systemically administered LPS has only systemic effects, whereas intratracheally administered LPS has minimal systemic manifestations. Local effects of LPS at other sites such as the gastrointestinal tract, spleen, and skin have been even less completely studied but should not be ignored in considering the overall host response to LPS.

From a practical perspective, especially within the framework of endotoxin effects in clinical infections and sepsis, LPS not only is injurious by itself but also augments other toxic agents so together they cause greater and more sustained damage. Thus, for example, LPS may boost the toxicity of ethanol and a variety of drugs of abuse. An alternative source of synergy is liver injury, which could potentiate deleterious LPS effects. Moreover, the potentially altered response to LPS in obesity or diabetes should be further explored. Little is known about the influence of aging on LPS effects, although proinflammatory effects of LPS manifest themselves differently in the aged population. Finally, gender influences on LPS action should be a fertile ground for studies, as sex hormones affect immune functions.

Tolerance versus Hypersensitivity to LPS

The influence of LPS on host cells and tissues is influenced by an individual's history. Tolerance or increased sensitivity to this agent may play a significant role in vivo. However, to discuss LPS hyporesponsiveness, LPS responsiveness needs to be defined. The following steps for LPS-macrophage interactions are a partial list: (i) interaction of LPS with appropriate LPS-binding proteins and then with an appropriate receptor, (ii) transmission of signalling through a membrane associated transducer protein, (iii) transmission of intracellular signals (i.e., tyrosine kinase activity is required and G proteins, and perhaps others, are involved), (iv) mobilization of nuclear signals such as NFκb, (v) gene induction, and (vi) release of soluble factors such as TNF, IL-1, and IL-6. The action of these factors on target cells ultimately leads to LPS sensitivity.

Under conditions in which the exposure to LPS is relatively modest, such as the normal response to gut-derived LPS, the inducible responses may maintain macrophages at a certain level of differentiation that contributes to innate resistance to infection and malignancy. In the response to gram-negative sepsis, however, the overwhelming stimulation of macrophages by LPS results in an uncontrolled systemic inflammatory response which is pathological. A breach in this cascade may attenuate the response. Thus, agents that block LPS from interacting with the macrophage may preclude, or at least diminish, the intensity of the initial signalling event. Agents that block intracellular signaling (i.e., genestein), preclude transcription of certain genes (i.e., glucocorticoids), or block the action of cytokines on target cells (anti-cytokine or anti-cytokine receptor antibodies or antagonists) will all disrupt the "cascade." Perhaps one of the shortfalls, to date, of any given strategy for the treatment of gram-negative sepsis is that virtually every study has focused on intervention at a single site in the cascade rather than attempting to block multiple sites simultaneously.

Two well-established models have provided insights into the cellular and molecular mechanisms that underlie LPS responsiveness. The first involves C3H/HeJ inbred mice, which resist the pathophysiological effects of even very high doses of LPS. Paradoxically, these mice are highly sensitive to infections with gram-negative bacteria, which may reflect a failure of C3H/HeJ macrophages to be stimulated by gut-derived LPS, thus reducing exogenous resistance to LPS.

The mouse gene controlling responsiveness to LPS is on chromosome 4 and is designated *Lps*, with two known alleles: Lps^n (normal) and Lps^d (defective). Several other strains also possess defects at the *Lps* locus, but how the noncomplementing defect in these strains compares with the C3H/HeJ defect is not known. A mutation in any gene of the cytokine cascade alters the LPS response pattern.

The second model of LPS hyporesponsiveness is "acquired" refractoriness to LPS, a phenomenon recognized nearly 50 years ago. Two types of inducible endotoxin tolerance have been defined. "Early" endotoxin tolerance is transient and is induced by exposure to sublethal doses of LPS. Following the "tolerizing" injection, responsiveness to LPS wanes over a 3- to 4-day period and then returns to normal. Tolerance is associated with specific and well-defined hematopoietic changes and a diminished capacity to produce cytokines after LPS challenge. The induction of tolerance to LPS appears to be mediated in part by cytokines, since rIL-1 and rTNF induce a state which resembles that induced by injection of a "tolerizing" dose of LPS. Moreover, if anti-TNF antibodies or rIL-1ra are administered to mice at the time of the tolerizing injection, tolerance is blocked.

"Late" endotoxin tolerance is long-lasting, O-antigen specific, and the result of production of anti-LPS antibodies. Unlike early tolerance, late phase tolerance is specific only for the LPS used to induce the tolerant state.

The nontoxic monophosphoryl lipid A (MPL) derivative can induce the early LPS refractory state, and clinical trials testing this approach in patients undergoing elective surgery are under way. When macrophage cultures are exposed to LPS and then challenged, cells fail to respond to the challenge dose of LPS to produce cytokines, like TNF. Apparently certain genes are actually "superinduced" in the desensitized macrophages.

These models of LPS hyporesponsiveness suggest several interventions in clinical sepsis.

Internalization and Catabolism of LPS

While LPS exerts profound effects on the host, the host also affects the biological and metabolic fate of LPS. Purified LPS binds to the surfaces of neutrophils and monocytes and then rapidly moves from the plasma membrane to intracellular sites. LPS passes at least transiently through endosomal (and possibly lysosomal) compartments, since raising the pH of these intracellular organelles decreases the rate at which LPS is degraded. A large fraction of cell-associated, purified LPS may return to the extracellular medium.

LPS bound to bacteria, in contrast, is internalized via (opsono)phagocytosis and surrounded intracellularly by the phagolysosomal membrane. Over time, fragments of bacterial membrane containing bioactive LPS may be released into the surrounding medium, and LPS is found on the surfaces of cells that have phagocytosed gram-negative bacteria.

Phagocytes can dephosphorylate and deacylate purified LPS in vitro, thereby reducing the macrophage-activating potential of LPS. However, these catabolic steps are so slow that the stimulatory potency of LPS is unlikely to be diminished. On the other hand, both dephosphorylation and partial deacylation produce metabolites that may inhibit the ability of LPS to stimulate cells. In human neutrophils, the major LPS-deacylating enzyme is not localized to any of the intracellular granules known to fuse with the phagolysosome, suggesting that LPS deacylation may occur in other intracellular compartments. Phospholipid metabolism proceeds slowly in phagocytosed bacteria and is carried out mainly by bacterial (not host) phospholipases. Moreover LPS in phagocytosed bacteria remains antigenic and immunogenic for weeks or perhaps even months, suggesting that the phagocytic vacuole is a poor site for LPS catabolism. Thus, the mechanism by which purified LPS is catabolized (inactivated) contrasts significantly with macrophage processing of bacterial cell wall LPS.

Antibody to Endotoxin and Immunotherapy

Endotoxins stimulate humoral immunity, another potential therapeutic tool for treating endotoxic shock. LPS antibodies occur in most mammalian species, including humans, following colonization or infection with gram-negative bacteria. The vast array and variety of such antibodies reflect both the complexity and phylogenetic diversity of LPS structure, as well as the ubiquitous nature of gram-negative bacteria in the endogenous and exogenous environment. Moreover, the functional specialization associated with different structural domains of the LPS macromolecule accounts, in part, for a corresponding functional diversity among antibodies that react with discrete LPS substructures. Modern techniques of analytical and synthetic chemistry as well as monoclonal antibodies (MAbs) that recognize defined LPS substructures are enhancing our understanding of the functional immunochemistry of the molecule.

LPS antibodies generally react with the phylogenetically heterogeneous, exposed portions of LPS on the bacterial cell surface, i.e., the O-side chain of phenotypically smooth LPS or the outer core of lipooligosaccharides (rough LPS). The antibacterial activity of these antibodies is closely related to their complement-activating ability and may be either phagocyte dependent, as in the case of opsonophagocytic activity, or phagocyte independent, as in the case of complement-mediated bacteriolysis.

The putative endotoxin-neutralizing properties of LPS antibodies are considerably more difficult to define. Late-phase tolerance is associated with the appearance of O-side chain-specific antibodies. Theoretically, antibodies directed against phylogenetically conserved, biologically active portions of the LPS macromolecule should provide cross-reactive, endotoxin-neutralizing, and cross-protective functions in systemic infections caused by diverse gram-negative bacteria. This idea has not proved easy to test or put into practice.

The functional role of antibodies in clearing LPS is poorly understood, despite its undoubted importance in host immunity to gram-negative bacterial disease. Immune complexes containing LPS and LPS-specific antibodies, for example, are opsonized by complement component C3b in the presence of serum, and the opsonized complexes are recognized by CR1 receptors on human erythrocytes and leukocytes. However, antibody-mediated LPS-cell interactions may have various physiologic consequences depending upon the type of cell, receptor(s), and corresponding signal transduction pathway(s) involved. Moreover, the cellular effects of LPS may be modified in critical ways by antibody-mediated LPS-cell interactions that interfere or compete with interactions that do not involve antibody.

The clinical potential—and pitfalls—of LPS MAbs as therapeutic agents in gram-negative bacterial disease are amply illustrated by recent clinical trials. Human and murine lipid A-specific MAbs lower the morbidity or mortality associated with gram-negative sepsis only in certain populations of patients. Therapy with antibody requires great attention regarding optimal patient selection, as well as potential mechanisms of action and optimal drug design.

The uneven antibacterial properties of LPS antibodies are puzzling. Why do antibodies to the LPS of certain organisms mediate complement-dependent bactericidal activity in the absence of phagocytic cells, whereas antibodies to LPS produced by other bacteria mediate only phagocyte-dependent killing? Further, although LPS-specific MAbs have been evaluated for in vitro bacterial killing, little is known about MAb-mediated in vivo killing. Improved animal models such as the canine model for septic shock would facilitate the evaluation of LPS antibody therapy in gram-negative bacterial disease.

Endotoxin, Endotoxemia, and Septic Shock: Caveats to the Concept

Endotoxin is not the inciting agent in all forms of irreversible shock. Gram-positive bacteria produce a clinical syndrome indistinguishable from gram-negative septic shock in the absence of endotoxemia. Even in gram-negative bacterial infections, factors other than endotoxin may significantly affect cardiovascular instability, organ damage, and outcome.

Although concentrations of circulating endotoxin correlate with higher mortality rates in some animal models of sepsis and in patients with meningococcemia, these studies do not establish causality. On the other hand, clinically important endotoxemia may oc-

cur when endotoxin is present but below detection levels or is sequestered at local sites of infection but releasing host mediators that exert systemic effects.

Induced tolerance, increased sensitivity, and genetic resistance to endotoxin do not necessarily improve the course of gram-negative infection in humans or animals. In C3H/HeJ mice, a normal response to endotoxin appears to protect against gram-negative infection. Proof that therapies specifically targeting endotoxins work in human septic shock is still lacking. □

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Suggested Reading

An extensive bibliography is available from the author.

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